Unraveling the auxin mechanism in 2,4-D induced somatic embryogenesis in Arabidopsis thaliana

Philipsen, C.

Chapter 1

The role of auxin in somatic embryo induction and development

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Abstract

Vegetative propagation of plants comes in many forms such as cuttings, and shoot- and root regeneration. Alternatively, somatic embryogenesis (SE) can be employed to generate stable clonal lines of the parents. In most protocols to induce SE, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is used to induce the redifferentiation of embryogenic cells from somatic cells. 2,4-D is a synthetic analogue of the natural plant hormone auxin, or indole-3-acetic acid (IAA). Auxin is well known for its instructive role in many plant developmental processes and growth responses such as embryogenesis, organogenesis and tropisms. However, the exact role of auxin in the complex process of SE is still not clarified. The molecular mechanism of SE and specifically how 2,4-D, and not IAA or other auxin analogues, is able to induce this process is not yet fully understood. Here the different forms of SE and the mechanism of auxin action are reviewed, with the aim to explain how the synthetic auxin analogue 2,4-D is able to induce totipotency in somatic cells, resulting in SE, and what are potential starting points to study this interesting developmental process.

Introduction

Each living organism, whether it belongs to the bacterial-, fungal-, plant-, or animal kingdom has its own way of reproducing. Two main routes of reproduction can be distinguished, either asexual resulting in offspring that is genetically identical to the parent, or sexual requiring the production of sexual cells, or gametes, through a process of reduction division, and the subsequent fusion of gametes. These gametes can be derived from the same (hermaphrodite) parent or from different (unisexual) parents (Roach et al., 2014). Unicellular organisms such as bacteria and fungi typically reproduce asexually, as their cells divide by binary fission or form daughter cells by budding (Attar, 2016). Several higher animal and plant species have adopted a natural asexual reproduction strategy, named parthenogenesis, where embryo development occurs independent of fertilisation. Parthenogenesis includes plant reproduction strategies such as apomixis, a collective naming for asexual reproduction in plants that involves fertilisation-independent embryo development from different parts of the ovule, and results in the formation of seeds carrying embryos identical to the female parent (Koltunow, 1993; Pupilli and Barcaccia, 2012). There are two distinct apomixis strategies in plants. In the first strategy no embryo sac is produced, and the embryo originates from one or more somatic cells of the maternal ovule tissue, the nucellus or inner integument. This strategy is commonly referred to as sporophytic apomixis or adventitious embryony. The second strategy entails the formation of an unreduced embryo sac, followed by embryo development from a diploid egg cell (gametophytic apomixis). In gametophytic apomixis, the unreduced embryo sac may arise from a somatic nucellar cell (apospory) or from an unreduced megaspore mother cell (diplospory) (for a detailed overview Pupilli and Barcaccia, 2012). Apomixis-loci have been identified in *Arabidopsis* and several other species, however, due to the complexity of the trait the exact role of genes controlling apomixis remains to be resolved (Barcaccia and Albertini, 2013).

In contrast, most higher plants and animals generally produce offspring with a unique genetic basis through sexual reproduction. The latter process involves zygotic embryogenesis, which starts with the establishment of the zygote by fusion of the male and female gametes. The embryo develops from the zygote by a combination of cell division and cell differentiation. The cell migration that is essential for animal embryogenesis does not occur in plant embryos, as plant cells are fixed in a cell wall matrix (Bedzhov et al., 2014; ten Hove et al., 2015). In the dicot model species *Arabidopsis thaliana* the basic body plan is established by a more or less programmed series of cell divisions forming respectively the

radial pattern, the apical-basal axis coinciding with the establishment of the shoot- and root apical meristem (SAM and RAM) stem cell zones (Figure 1a), and finally with the initiation of the cotyledon primordia the bilateral symmetry that is typical for dicot plant embryos (Meyerowitz, 2002). In the late immature zygotic embryo (IZE) the SAM is flanked by two cotyledons and connected with the basal RAM by the hypocotyl (Figure 1b, Lau et al., 2011). After germination, the final shape of the plant is elaborated from this basal body plan by organ formation from the SAM and from the RAM-produced pericycle layer during post-embryonic development (Figure 1c,d). This final plant shape can differ considerably depending on the environmental conditions under which plants are grown. It is this stem cell zone-driven flexibility in development that allows sessile plants to adapt to sudden changes in environmental conditions. Moreover, the regenerative capacity of plant cells, especially those in the stem cell zones, has allowed the development of *in vitro* systems for clonal propagation of crop plants. Plant clonal propagation can be readily achieved in tissue culture either through stem cuttings, or by shoot- and subsequent root regeneration from tissues requiring (*de novo)* organogenesis, or by the induction of somatic embryos. The latter process requires the induction of totipotent cells from somatic cells, in order to develop somatic embryos that will contain a root-shoot axis, a vascular system and functional meristems, all produced in one single developmental step and referred to as somatic embryogenesis (SE).

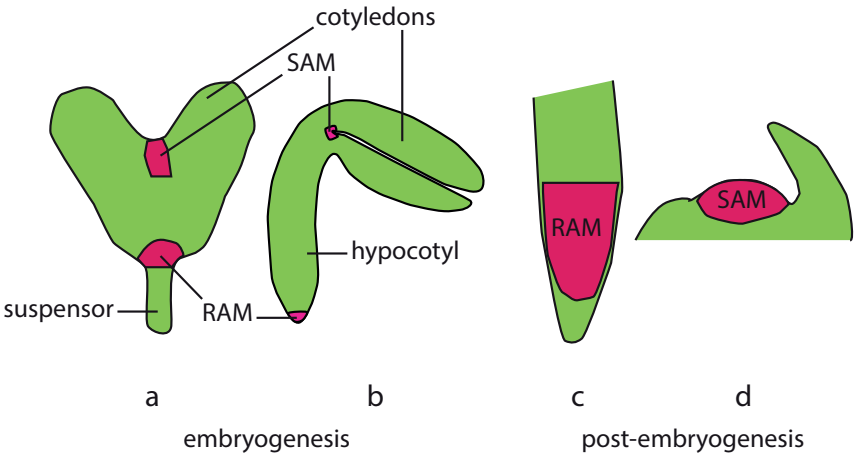

FIGURE 1 - Schematic overview of stem cell niches in Arabidopis embryos and post-embryonic tissues.
In the heart stage (a) and bent cotyledon stage embryos (b) the shoot apical meristem (SAM) is flanked by two cotyledons, whereas the root apical meristem (RAM) connects the embryo with the suspensor (a) or is located basally of the hypocotyl in the IZE (b). During post-embryonic development, the stem cell niches are located in the RAM (c) or in in the SAM of stems or leaves (d).

Both SE and apomixis are favourable in agriculture as a reproductive strategy for stable clonal propagation of heterozygous F1 crop plants, which allows for selecting gene combinations such as those showing heterosis (Pupilli and Barcaccia, 2012). Other applications include propagation of open pollinated crops, crops with long life cycles, or male sterile lines (Pupilli and Barcaccia, 2012; Barcaccia and Albertini, 2013). Propagation via SE has a number of advantages over other forms of asexual propagation or *in vitro* organogenesis in that cell cultures can be immortalized and scaled-up in bioreactors and the resulting embryos can be cryopreserved to store for later use or to establish gene banks (Timmis, 1998; Arnold et al., 2002). Moreover, as opposed to shoot/root regeneration, SE has been reported to result in limited somaclonal variation among the propagated individuals (reviewed by Gaj, 2004; Bairu et al., 2011) and can be induced on multiple types of explants, generally by using high concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), a synthetic analogue of the plant hormone auxin. In this Chapter the auxin mechanism is introduced and our current knowledge on 2,4-D induced SE and transcription factor induced SE is reviewed.

The plant hormones auxin and cytokinin in plant clonal propagation

Based on early plant tissue culture experiments, the plant hormones auxin and cytokinin have been identified as key regulators of plant regeneration. Natural cytokinins identified are adenine derivates that induce cell division and are involved in phyllotaxis and many other developmental processes (Kieber and Schaller, 2014). The first cytokinin identified, kinetin, was found in autoclaved herring sperm DNA as a potent activator of tobacco cell proliferation. In the same laboratory, another purine derivative, 6-benzylaminopurine (BA) was discovered and shown to stimulate cell division in cultured tissues (Miller et al., 1955, 1956). The most abundant naturally occurring cytokinin in plants, zeatin, was isolated for the first time from maize (Letham, 1963).

The discovery of auxin as a plant growth regulator was triggered in the late 19th century by experiments of Charles Darwin and his son Francis on the phototropic bending of oats coleoptiles (Darwin and Darwin, 1880). Following the initial isolation of the causative agent responsible for this response by Fritz Went in the 1920s, and its identification as indole-3-acetic acid (IAA) a few years later (Kögl et al., 1934), several other auxinic compounds have been identified, such as the naturally occurring indole-3-butyric acid (IBA) and the synthetic auxin analogue naphthalene acetic acid (NAA). Until now, only four naturally occurring compounds with auxin activity have been described in plants: IAA, IBA, 4-chloroindole-3-acetic acid (4-CL-IAA) and phenylacetic acid (PAA). All four are low molecular weight organic acids containing an aromatic ring (an indole

or phenyl group) with a carboxyl group attached. Over the years, compounds, such as 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), naphthalene-1-acetic acid (1-NAA), 4-amino-3,5,6-trichloropicolinic acid (picloram), and 2-methoxy-3,6-dichlorobenzoic acid (dicamba) were discovered to exhibit auxin-like activity (De Rybel et al., 2009; Sauer et al., 2013). Based on their toxicity at high concentrations especially for dicot plants, 2,4-D and 2,4,5-T have emerged as successful selective herbicides during the second world war (Hamner and Tukey, 1944). Because of their plant growth regulating properties and stability in tissue culture synthetic auxins have provided a useful tool to study the effects of auxin response, -homeostasis and –transport in a diverse range of plant developmental processes. Each type of auxin can result in a different developmental response depending on its concentration and the tissue it is applied to (Simon et al., 2013), resulting in growth, organogenesis, callus formation or even in SE. By controlling the ratio of auxin and cytokinin in the culture medium, either shoots, callus, or roots can be produced from the original tissue explants (Murashige and Skoog, 1962). Besides inducing organogenesis, high concentrations of the synthetic auxin 2,4-D, in several cases combined with cytokinin, can also induce SE. Hormone-induced SE was documented first in carrot cell suspension cultures (Steward et al., 1958). Since then, the production of somatic embryos has been described for various plant species, and a few model systems for SE have emerged as well as successful combinations of hormones that induce SE. In conifers 2,4-D is combined with BA (von Arnold et al., 2002; Filonova et al., 2000) and in *Medicago truncatula* SE is induced with NAA and BA (Wang et al., 2011). In the monocot model system rice, SE is induced with 2,4-D and kinetin (Verma et al., 2011; Bevitori et al., 2013) and in *Arabidopsis thaliana* 2,4-D alone is sufficient to induce SE (Luo and Koop, 1997; Gaj, 2001; Rose and Nolan, 2006; Nowak et al., 2012). To study SE in dicots, the *Arabidopsis* SE system is useful, due to its large number of available explants, high efficiency and the availability of ecotypes, reporter lines, and knock-out and overexpression mutants.

Arabidopsis somatic embryogenesis

In *Arabidopsis* SE can be induced by 2,4-D on immature zygotic embryos (IZEs), protoplasts, meristems, shoot apical tip and floral bud explants (Luo and Koop, 1997; Mordhorst et al., 1998; Gaj, 2001; Ikeda-Iwai et al., 2003). In addition, SE can be induced on IZEs or germinating seedlings in the absence of 2,4-D by the ectopic expression of specific transcription factors, such as the embryo maturation gene *LEAFY COTYLEDON 1* (*LEC1*) (Lotan et al., 1998), the shoot meristem organizer *WUSCHEL* (*WUS*) (Zuo et al., 2002), or the early embryo fate gene *BABYBOOM* (*BBM*) (Boutilier et al., 2002).

Here I review the recent advances in how 2,4-D possibly controls the SE process at the genetic, molecular and cellular levels and compare it to transcription factor induced SE to identify common targets for this complex regeneration process.
SE induction can be established via a 1-step or 2-step protocol on *Arabidopsis* IZEs. In the 1-step protocol, generally, the IZE is 14 days cultured on 2,4-D-containing medium, followed by transfer to hormone free medium for 7 days (Gaj, 2011). In this 1-step protocol, somatic embryos are considered to either develop directly from the explant and have a unicellular origin (direct SE, DSE), or to develop from a callus and thus have a multicellular origin (indirect SE, ISE) (Kurczyńska et al., 2007). Alternatively, the 2-step protocol SE is initiated by a culture period of 24 days on different types of 2,4-D-containing media, followed by a culture period on hormone free medium (Su et al., 2009; Nowak et al., 2012). In this second protocol, embryogenic calli are first established, and will only produce somatic embryos through ISE. This process is also referred to as secondary, recurrent or repetitive SE (Mordhorst et al., 1998). The protocol developed for obtaining *Picea abies* somatic embryos is exemplary: proembryogenic masses (PEMs) are first established using a mix of 2,4-D and BA, after which maturation of somatic embryos is induced by replacing 2,4-D and BA with the maturation-inducing hormone abscisic acid (ABA) (Timmis, 1998). The *Picea* PEM cultures can be cryopreserved after a period of culture on lower 2,4-D and BA concentrations, which has not been shown yet to work in *Arabidopsis*. In all described protocols for SE induction, 2,4-D is the main inducer of SE leading to cell proliferation, dedifferentiation and eventually to the initiation of somatic embryos.
In the *Arabidopsis* 1-step protocol, an initial swelling can be observed at the macroscopic level after 3 days on the abaxial and adaxial side of the cotyledons of IZEs, at which time the IZEs have unfolded from a bent cotyledon (Figure 2a) towards a "Y" shape, probably as a response to the 2,4-D treatment (Figure 2a) (Raghavan, 2004; Bassuner et al., 2006). The first extensive histological analysis of somatic embryos in *Arabidopsis* showed that the first mitotic divisions are visible as early as 2 days in culture, starting in the procambial cells at the adaxial side of the cotyledon base (Raghavan, 2004). Within 3-4 days of culture, cell divisions in the procambial cells can also be observed on the abaxial side and in epidermis and mesophyll cells on both abaxial and adaxial sides of the cotyledon (Figure 2b). Initial divisions and a higher number of cell layers are observed in the adaxial side, but the same processes can be observed on the abaxial side. Cell divisions spread through both cotyledons resulting in callus in the original mesophyll (Raghavan, 2004). The earlier study by Raghavan does not distinguish between DSE and ISE, but does describe globular embryonic structures on the epidermal cells on the adaxial side. In a later analysis, DSE and ISE were distinguished based on the origin of the cell forming the somatic embryo (Kurczynska et al. 2007) (Figure 2b,c). The ISE events from multicellular origin

are derived from both protodermal (epidermis) and subprotodermal (subepidermis) cell layers; however, a detailed analysis of the development of a somatic embryo derived through ISE is still lacking. In contrast to the earlier description of cell divisions by Raghavan (2004), Kurczyńska and coworkers (2007) state that callus mostly develops on the abaxial side, suggesting ISE preferentially occurs on this side (Figure 2b). Kurczynska and colleagues (2007) describe DSE events that can be recognized by single protodermal cells becoming enlarged and subsequently dividing periclinally and anticlinally. Additionally, multicellular embryonic structures can be observed as well. These originate from both protodermis and subprotodermis layers with similar periclinal and anticlinal divisions on the adaxial side (Figure 2c). The multicellular structures can also be recognised by thicker cell walls outlining the presumed early somatic embryos (Kurczyńska et al., 2007).

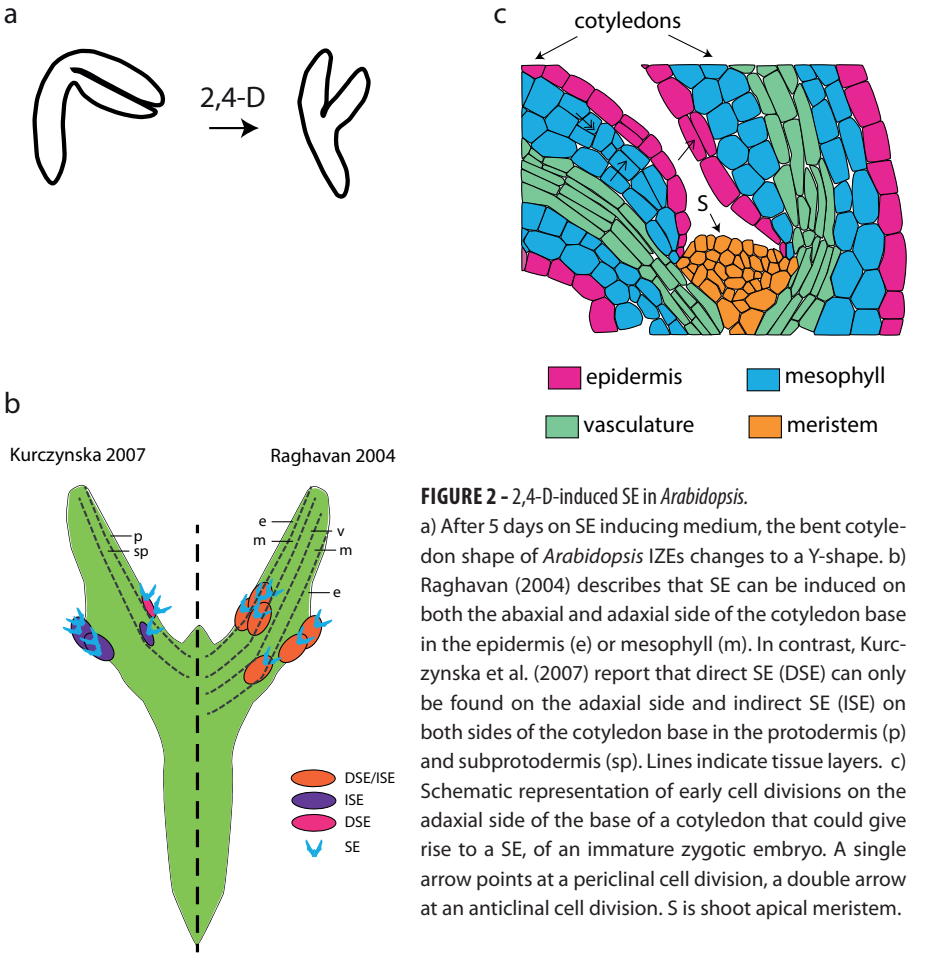

FIGURE 2 - 2,4-D-induced SE in *Arabidopsis*.
a) After 5 days on SE inducing medium, the bent cotyledon shape of *Arabidopsis* IZEs changes to a Y-shape. b) Raghavan (2004) describes that SE can be induced on both the abaxial and adaxial side of the cotyledon base in the epidermis (e) or mesophyll (m). In contrast, Kurczynska et al. (2007) report that direct SE (DSE) can only be found on the adaxial side and indirect SE (ISE) on both sides of the cotyledon base in the protodermis (p) and subprotodermis (sp). Lines indicate tissue layers. c) Schematic representation of early cell divisions on the adaxial side of the base of a cotyledon that could give rise to a SE, of an immature zygotic embryo. A single arrow points at a periclinal cell division, a double arrow at an anticlinal cell division. S is shoot apical meristem.

When somatic embryos are left to develop for 14 to 21 days on 2,4-D-containing medium followed by a hormone free period, they resemble zygotic embryos without a vascular connection to the original IZE or callus (Raghavan, 2004; Bassuner et al., 2006; Kurczyńska et al., 2007). In the 1-step protocol 73-90% of the explants used can successfully induce SE, producing on average 2 to 10 somatic embryos with normal (also described as "true"), trumpet-shaped or fused cotyledons (Gaj, 2001). It has been reported that "true" somatic embryos are attached to the IZE via a file of cells similar to a suspensor (Luo and Koop, 1997), although no evidence of suspensor identity has been provided yet. The development of somatic embryos with fused cotyledons or multiple somatic embryos fused together at the shoot to root tip complicates research on SE when determining how efficient a particular genotype is in SE. Scoring methods are mostly based on the number of somatic embryos produced per IZE (SE productivity) (Luo and Koop, 1997) and recognizing a "true" somatic embryo is proven to be difficult.

A recurrent problem in histological analysis of regenerating plant tissues is the early identification of which cells will develop into embryos, roots, shoots or callus. For example, there is insufficient evidence that the previously described thickened cell wall is only surrounding those cells that will develop into a true somatic embryo. Despite detailed histological analysis, it is therefore still difficult to draw comprehensive conclusions on the origin and preferred occurrence of somatic embryos, in part because of differences in the definitions and terms used in the above studies. Based on the two studies one can deduce that somatic embryos originate from both epidermal and mesophyll tissues, and that the somatic embryos derived from the epidermal layers are most likely a consequence of DSE. The use of cell fate specific markers and an accurate quantification of DSE and ISE events would clarify on how often and where in the IZE, DSE and ISE mostly occur.

By comparing SE with systems that use organogenesis for propagation extract a few similarities can be extracted. Similar to SE, organogenesis is established from somatic cells directly or indirectly via a callus phase. Direct organogenesis involves regeneration without a callus phase and includes the reprogramming of cells e.g. adventitious root formation (Verstraeten et al., 2014). Indirect organogenesis includes the regeneration of roots and shoots via a callus phase and can be divided into two phases. Firstly, somatic cells need to respond to exogenously applied hormones and subsequently redifferentiate to a totipotent cell state. Secondly, these cells (callus) are reprogrammed and assigned with a specific cell fate and thirdly, the completion of organ regeneration (Su and Zhang, 2014; Kareem et al., 2015). The cell divisions as described by Raghavan (2004) during SE are similar to those observed during callus- and lateral root

initiation. Assuming that ISE events result from the cell divisions in layers around the vasculature, it can be considered similar to the divisions of pericycle cells, a layer of cells surrounding the root vasculature giving rise to the lateral root (described and reviewed by De Smet 2012). Both lateral roots and somatic embryos will have to push through a layer of respectively cortex- or mesophyll cells and the epidermis. Molecular and genetic regulation enabling this development might overlap, as was shown for callus development from roots and the lateral root program (Sugimoto et al. (2010). The root identity of root-derived callus might be the key difference with embryogenic callus, of which it is assumed to be an undifferentiated mass of totipotent cells (Sugimoto et al., 2010; Sena and Birnbaum, 2010).

2,4-D, a common SE inducer

As indicated above, the most common method to induce SE is exposure of explants to synthetic derivatives of the plant growth regulator auxin, such as NAA, 2,4-D, 3,6-dichloro-2-methoxybenzoic acid (dicamba), or 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) (Jiménez 2005). The importance of the synthetic auxin 2,4-D for the induction of SE was recognized more than 50 years ago (Steward et al., 1958; Halperin and Wetherell, 1965), and the vast majority of SE protocols, including those for *Arabidopsis*, use 2,4-D as the inducing agent (Gaj, 2001). To be able to understand its action the different components in the auxin homeostasis, transport and response pathways are introduced.

Auxin homeostasis

Typical for a hormone, the action of auxin is on the one hand determined by its concentration in the cell, which is a product of transport, biosynthesis and metabolism. On the other hand, auxin can only act if cells are sensitive to the hormone. The auxin response pathway, involving hormone receptors and downstream transcriptional regulators, determines this sensitivity. Mutant screens in *Arabidopsis thaliana* have revealed most of what it is known from the auxin pathway.

IAA is produced in meristems, young primordia, vascular tissues and reproductive organs and research in *Arabidopsis* and *Zea mays* has uncovered several IAA biosynthesis routes, which fall into two categories: i) the tryptophan (Trp)-dependent and ii) the Trp-independent routes (Woodward and Bartel, 2005). In here, I focus on the Trp-dependent route. Four Trp-dependent pathways have been uncovered and are named after the intermediate compound produced

using L-tryptophan (Trp) as a substrate: 1) the indole-3-acetaldoxime (IAOx) pathway, 2) the indoleacetamide (IAM) pathway, 3) the tryptamine pathway (TAM) and 4) the indole-3-pyruvic acid (IPyA) pathway (Figure 3) (Woodward and Bartel, 2005; Korasick et al., 2013; Ljung, 2013).

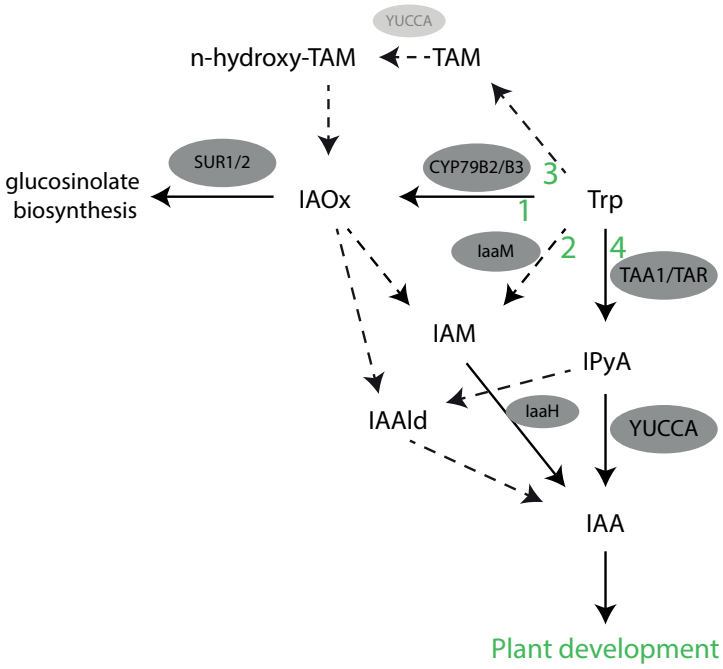

FIGURE 3 - A schematic overview of the tryptophan (Trp)-dependent auxin biosynthesis pathways.
Four Trp-dependent pathways have been suggested in the production of indole-acetic acid (IAA). (1) indole-3-acetaldoxime (IAOx) is produced from Trp by Cytochrome P450 monooxygenases CYP79B2 and CYP79B3 and can be used to produce glucosinolates by SUPERROOT1 and 2 (SUR1/2). IAOx may also be converted to indole-3-acetaldehyde (IAAld) and indoleacetamide (IAM), from which IAA is produced. (2) IAM may be produced directly from Trp by Trp monoxygenase (IaaM), which is than converted to IAA by IAM hydrolase (IaaH). (3) The conversion to tryptamine (TAM) and to n-hydroxy TAM, is possibly catalyzed by YUCCAs and results in IAOx. (4) Trp is converted to indole-3-pyruvic acid (IPyA) by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 / TRYPTOPHAN AMINOTRANSERASE RELATED 1 TO 4 (TAA1/TAR1-4) and subsequently to IAA by YUCCAs. Solid arrows are identified pathways, dashed arrows have not been confirmed yet and may have one or multiple steps. The scheme is based on Woodward and Bartel (2005), De Paepe et al. (2005), Korasick et al. (2013), Nishimura et al. (2014), He et al. (2001), Ljung (2013).

Typical phenotypes that results from mutants overproducing endogenous auxin are seedlings with epinastic cotyledons, elongated petioles, a shorter root, a more branched root system, or more and longer root hairs (Zhao et al., 2001). These typical auxin overproduction phenotypes were observed in mutants that ectopically express *CYP79B* as well as in knock out mutants of *SUPERROOT1 (SUR1)*

and SUR2 (Boerjan et al., 1995; Delarue et al., 1998). IAOx is produced from Trp by the Cytochrome P450 monooxygenases CYP79B2 and CYP79B3. Accumulation of IAOx by *CYP79B2* overexpression or by blocking glucosinolate biosynthesis via *SUR1* and *SUR2* disruption results in auxin overproduction, which explains the observed phenotypes (Zhao et al., 2002; Sugawara et al., 2009*;* Boerjan et al., 1995; Delarue et al., 1998) (Figure 3.1).

In the *cyp79b2 cyp79b3* double mutant, IAM levels are decreased, suggesting that IAM is likely made from IAOx (Sugawara et al., 2009). However, based on the auxin production pathway identified in the bacteria *Agrobacterium tumefaciens* and *Pseudomonas syringae* it has been hypothesized that IAM may also be produced directly from Trp by Trp monoxygenase (IaaM) (Figure 3.2). A second bacterial enzyme IAM hydrolase (IaaH) is known to convert IAM to IAA (Woodward and Bartel, 2005); however, whether this pathway exists in plants still needs further confirmation. The second possible mechanism to convert Trp to IAA is by conversion to tryptamine (TAM) (Figure 3.3). In mammalian systems, FLAVIN MONOOXGENASEs (FMOs) convert TAM to N-hydroxy-TAM (Zhao et al., 2001). A family of 11 FMO-like enzymes identified in *Arabidopsis* were proposed to fulfil a similar role and are now known as the YUCCA (YUC) family of proteins (Zhao et al., 2001; Cheng, 2006). N-hydroxy-TAM would be then converted to IAOx, followed by indole-3-acetaldehyde (IAAld) and finally to IAA (Woodward et al., 2005; Stepanova et al., 2011). However, evidence came forward that IAOx is not likely produced by YUCs (Sugawara et al., 2009) and later evidence has placed YUCs downstream of TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 / TRYPTOPHAN AMINOTRANSERASE RELATED 1 TO 4 (TAA1/TAR1-4) family enzymes in the IPyA pathway (Stepanova et al., 2011; Won et al., 2011) (Figure 3.4). TAA1/TAR converts Trp into indole-3-pyruvic acid (IPyA), and YUCs subsequently produce IAA from IPyA by oxidative carboxylation. Together these enzymes constitute the most well researched auxin biosynthesis pathway in plants, the IPyA pathway (Won et al., 2011; Ljung, 2013; Korasick et al., 2014).

The *TAA1* gene was first discovered by study of the *wei8* loss-of-function mutant, which showed a defective ethylene response in roots. Defects in *TAA1/SHADE AVOIDANCE3/WEAK ETHYLENE INSENSITIVE8/TRANSPORT INHIBITOR RESPONSE2* (*TAA1/SAV3/WEI8/TIR2*) results in decreased free IAA (Stepanova et al., 2008; Tao et al., 2008). Since the defect could be reversed by exogenous IAA application, the TAA1 enzyme was thought to act as an aminotransferase that converts Trp into IPyA (Stepanova et al., 2008). Mutations in combinations of *TAA1* with its closest homologs *TAR1* and/or *TAR2* results in altered meristem functioning, and in defects in flowering and embryo development (Stepanova et al., 2008).

The *wei8 tar1 tar2* triple mutants fail to develop the basal part of the embryo (Stepanova et al., 2008) similar to quadruple *yuc1 yuc4 yuc10 yuc11* mutants (Cheng, 2006; Cheng et al., 2007). Based on these results the TAA1/TAR – YUCCA pathway is now considered as the most important pathway in higher plants for auxin production (Figure 5.2).

Besides free IAA, which is the active hormone, auxin can be present in the cell in several inactive storage forms. IAA can be conjugated to sugars (e.g. IAA-Glucose), amino acids (IAA-Ala, IAA-Leu, IAA-Asp, IAA-Glu, IAA-Trp), or it can be converted to other natural auxins, such as IBA (Figure 4.2) or to the inactive methyl ester form (MeIAA). IAA is converted to IBA by IBA synthase in microsomal membranes (Ludwig-Muller et al., 1995) and its conversion back to IAA is located in the peroxisome (Zolman et al., 2000; Strader et al., 2010). Most of described storage forms can be converted back to active IAA, but some forms (IAA-Asp, IAA-Glu and oxIAA) are permanently storing auxin in an inactive form (for a detailed review read Korasick et al., 2013). The role of IBA as either a storage or active form is still unclear, although it has been suggested to be a stable transport form of IAA. Long distance IBA transport was shown both in basipetal and acropetal directions similar to IAA, but how exactly remains uncharted (Simon and Petrášek, 2011; Strader and Bartel, 2011). IBA research deserves more attention as IBA constitutes 25-30% of the total auxin pool in Arabidopsis seedlings (Ludwig-Muller et al., 1993), and the compound is commonly used as a root inducing growth hormone in tissue culture.

Auxin transport

For IAA it is well-established that it is directionally transported in the plant from sites of biosynthesis to sites of action, and that this cell-to-cell transport is facilitated by in- and efflux carriers. In the 1970s, the chemiosmotic hypothesis for polar auxin transport (PAT) was proposed (Rubery and Sheldrake, 1974; Raven, 1975), which states that in the weakly acidic apoplast, 15% of the IAA molecules are protonated (IAAH) and these molecules can enter the plant cell by diffusion. The majority of the IAA molecules, however, are in the deprotonated form (IAA^-) and require active uptake by influx carriers (Figure 4.1). Once inside the more basic cytosol, the protonated auxin molecules become deprotonated, and these deprotonated auxin molecules cannot pass the membrane by diffusion, preventing auxin efflux. However, auxin efflux carriers with an asymmetric plasma membrane distribution can facilitate auxin export at one side of the cell where the partially protonated auxin can be taken up by the neighbouring cell (Swarup and Péret, 2012; Petrásek and Friml, 2009).

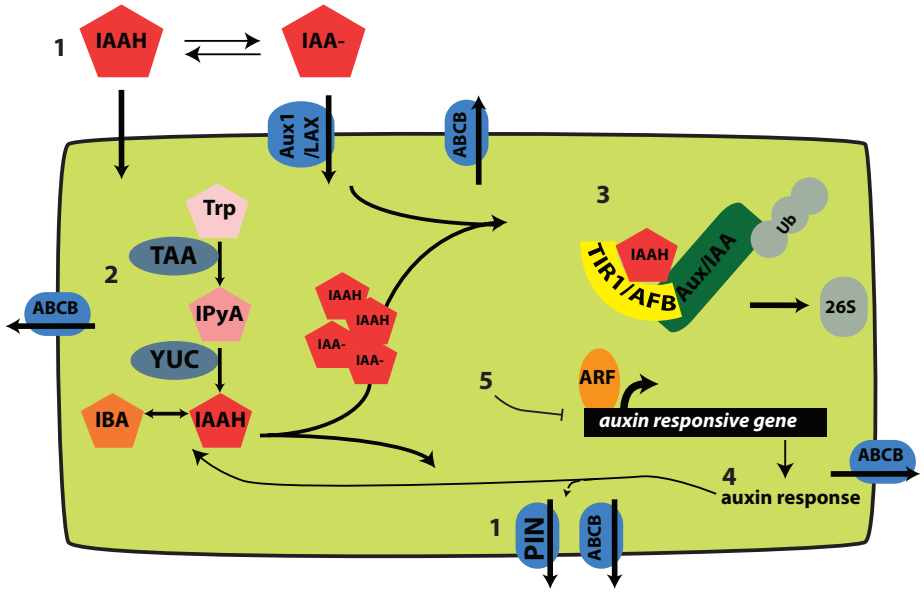

FIGURE 4 - Schematic overview of auxin homeostasis, transport and signaling in *Arabidopsis*.
Auxin homeostasis is established by 1) passive uptake of protonated IAA (IAAH) via the plasma membrane or active uptake of deprotonated IAA (IAA-) by AUX1/LIKE-AUX1 (AUX1/LAX) import carriers and efflux by PIN and ABCB export carriers. 2) Auxin is synthesized from tryptophane (Trp) to indole-3-pyruvic acid (IPyA) by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINOTRANSFERASE RELATED (TAA1/TAR) family enzymes. The final product, IAA, can be converted to the transport form indole-butyric acid (IBA) or to conjugated storage forms. Auxin signaling is established by 3) IAA-dependent recruitment of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) repressor proteins by the TRANSPORT INHIBITOR RESPONSE1/AUXIN RECEPTOR F-BOX PROTEINS (TIR1/AFB) E3 ubiquitin ligase complex, resulting in their ubiquitination and subsequent degradation by the 26S proteasome. This releases the repression of AUXIN RESPONSE FACTOR (ARF) mediated initiation of 4) auxin responsive gene expression, which in turn may enhance auxin biosynthesis or transport, and eventually result in 5) negative feed back by the enhanced production of the Aux/IAA repressor proteins.

Research in *Arabidopsis* has identified candidate proteins for these auxin influx and efflux carriers. Auxin influx is thought to be mediated by the AUX1/LAX family of transporters, comprising the four highly conserved AUX1 and LIKE-AUX1 (LAX) proteins LAX1, LAX2 and LAX3 (Swarup and Péret, 2012). Based on auxin transport measurements in *Arabidopsis* inflorescence stems, AUX/LAX proteins are key drivers of polar auxin transport, as they load auxin into the transporting cells (Boot et al., 2016). They are known for their involvement in several developmental processes, such as gravitropism, lateral root development, vascular patterning, phyllotaxis, and embryogenesis (Swarup and Péret, 2012; Robert et al., 2015). Auxin efflux carriers have been identified through the *Arabidopsis pin formed* mutant that develops pin-like inflorescences, a phenotype that pheno-

copies treatment of *Arabidopsis* plants with inhibitors of PAT. The *Arabidopsis* genome encodes a small family of eight PIN proteins that both have overlapping and different roles in plant development (Friml et al., 2002; Benková et al., 2003; Friml et al., 2003; Blilou et al., 2005). PIN proteins consist of two regions of transmembrane domains interrupted by a hydrophilic loop (PINHL), that has recently been mapped to localize in the cytosol (Nodzyński et al., 2016). PINs with a long cytosolic loop (long PINs) are localized on the PM (PIN1, 2, 3, 4 and 7), whereas PINs with a short cytosolic loop (short PINs) are localised in the endoplasmic reticulum (ER) (PIN5 and PIN8). PIN6 contains a hydrophilic loop of intermediate length and is localised both in the ER and at the PM (Mravec et al., 2009; Sawchuk et al., 2013; Ganguly et al., 2014). Asymmetric localisation of PINs results in local dynamic auxin maxima and minima or gradients that are instructive in many developmental processes, including embryo patterning (Friml et al., 2003; Robert et al., 2015), tropic growth (Luschnig et al., 1998; Friml et al., 2002) and organ formation (Reinhardt et al., 2000; Benková et al., 2003). All the long PINs have been shown to be involved in patterning the zygotic embryo by generating dynamic auxin maxima that first mark the early embryo proper, then establish the root pole, and finally position the cotyledon primordia (Friml et al., 2003; Blilou et al., 2005).

In addition to the PIN proteins, three ABC transporters have been identified, respectively ABCB1, ABCB4 and ABCB19, that function in PAT as well, (Noh et al., 2001; Santelia et al., 2005; Cho et al., 2007). They are distributed more symmetrically than PIN proteins in the plasma membrane (Figure 4.1). They may facilitate non-polar IAA efflux to contribute to long-distance IAA transport, and thereby controlling the auxin availability to PAT (Mravec et al., 2008; Geisler and Murphy, 2006; Titapiwatanakun et al., 2009). Neither of these efflux proteins has been reported to transport other forms of auxin, besides IAA.

Several lines of evidence reveal the significance of auxin biosynthesis and transport for plant development. *Arabidopsis* seedlings with defects in combinations of YUC1, YUC2, YUC4, YUC6 and PIN1 show developmental abnormalities in embryo, leaf and flower development (Cheng et al., 2007). These are seen as the key steps in regulating organogenesis (Cheng, 2006; Stepanova et al., 2008; Robert et al., 2013; Nishimura et al., 2014). Furthermore, Robert and colleagues (2013) have shown that polar transport of localized auxin production by the IPyA pathway is necessary to establish the embryonic apical-basal axis in a spatio-temporal manner. These results illustrate the need for a well-established auxin gradient to control cell patterning and differentiation. Through the combined action of these transporters and local auxin production, dynamic auxin maxima and minima can be formed that are involved in patterning and organ formation

through cell division and differentiation (Bohn-Courseau, 2010; Marhavý et al., 2013; Xuan et al., 2015)

Auxin Response

Auxin is perceived in the nucleus to activate auxin responsive gene expression by the AUXIN RESPONSE FACTORS (ARFs) (Figure 4.3). The *Arabidopsis* genome encodes 23 ARFs (Guilfoyle and Hagen, 2007) of which five have been classified as transcriptional activators and the remaining as repressors based on the amino acid sequence in the middle region of the ARF protein (Tiwari et al., 2003). The transcriptionally activating ARFs possess a glutamine-enriched middle region that acts as activation domain (AD), whereas repressing ARFs have P-,S-, and T-rich middle region. On the N-terminal side of the middle region, a B3 DNA-binding domain (DBD) is flanked by two dimerization domains (DD) (Guilfoyle, 2015; Wright and Nemhauser, 2015; Adams-Cioaba et al., 2012). The DDs facilitate binding of ARFs as dimers to the auxin response elements (AuxREs) in the promoters of auxin responsive genes. The identification of AuxREs in many promoter regions has allowed deducing the TGTCTC and TGTCGG consensus sequences and their use to generate the artificial *DR5* and *DR5v2* promoters, respectively. These promoters have been abundantly used to monitor auxin responses in plants during cellular responses, cell- and tissue development (Ulmasov et al., 1997; Boer et al., 2014; Liao et al., 2015).

When the auxin concentration in the cell is low, transcription of auxin responsive genes is repressed by the pairing of the activating ARFs with one of the 29 family members of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) repressor proteins in *Arabidopsis*. Aux/IAA proteins have initially been identified as the products of primary auxin response genes (Overvoorde et al., 2005). Most Aux/IAAs consist of three conserved domains: a conserved C-terminal domain that interacts with ARFs, an N-terminal domain I (DI) that recruits co-repressor proteins like TOPLESS (TPL) and is required for transcriptional repression, and the middle domain II (DII) that determines Aux/IAA protein instability through its auxin-enhanced interaction with the TRANSPORT INHIBITOR RESPONSE1/AUXIN RECEPTOR F-BOX PROTEINS (TIR1/AFB) auxin co-receptors (Wang and Estelle, 2014).

The *Arabidopsis* genome encodes 6 TIR1/AFBs proteins, which are F-box proteins that are part of $SCF^{TIR1/AFB}$ E3 ubiquitin protein ligase complexes. Auxin binding to their hydrophobic pocket stabilizes the TIR1/AFB – Aux/IAA co-receptor complex and allows the SCF^{TIR} E3 ubiquitin ligase to ubiquitinate the Aux/IAA proteins, which again targets these repressor proteins for degradation by the 26S proteasome (Maraschin et al., 2009; Dharmasiri et al., 2005a, 2005b; Parry et al., 2009). Degradation of Aux/IAA proteins releases the transcriptional repression and

allows the ARFs to activate or inactivate transcription of auxin responsive genes (Figure 4.3). The fact that the *Arabidopsis* genome encodes in total 6 TIR1/AFBs, 29 Aux/IAAs and 23 ARFs, hypothetically allows 4002 possible combinations of interactions which may lead to the same number of possible responses. In reality, however, the interactions are dependent firstly on the specific binding affinity of each TIR1/AFB for the same Aux/IAA protein (Calderón Villalobos et al., 2012; Parry et al., 2009). Secondly, each co-receptor pair has its own binding affinity for auxin, which is dependent on the efficiency (whether the molecule will bind) and specificity (the structure of the pocket) of the complex (Lee et al., 2014). Although it has been established that synthetic auxin analogues can bind the TIR1/AFB – Aux/IAA co-receptor pairs with high affinity, some analogues were shown to have higher affinity for certain co-receptor pairs, e.g. the synthetic auxin picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) has a higher affinity for the AFB5-Aux/IAA than TIR1-Aux/IAA co-receptors (Lee et al., 2014). Thirdly, an additional layer of complexity is provided by the fact that Aux/IAAs can interact with different and multiple ARFs (Figure 5). Therefore, the final response of plant cells to auxin will thus be determined by the type of auxin, the binding interaction of TIR1/AFB – Aux/IAA co-receptors with an auxin molecule and the interaction between Aux/IAAs and ARFs that will activate auxin responsive gene expression (Piya et al., 2014; Korasick et al., 2014; Bargmann et al., 2013). Auxin responsive gene expression may in turn result in the redistribution of PIN proteins (Baster et al., 2013) (Figure 4.4), or in stimulating auxin biosynthesis and metabolism (Xuan et al., 2015), which, through a negative feedback loop, can decrease the auxin response by inducing production of extra Aux/IAA proteins (Figure 4.5).

Besides the nuclear TIR1/AFB – Aux/IAA co-receptor complexes, several lines of evidence indicate the existence of other receptors that mediate rapid responses to auxin that do not necessarily require gene transcription, such as the rapid increase in cytosolic calcium ions ($[Ca^{2+}_{cyt}]$) (Monshausen et al., 2011), or the immediate inhibition of PIN endocytosis (Paciorek et al., 2005; Robert et al., 2010) upon auxin treatment. One of them is S-phase-kinase- associated protein 2 (SKP2), which is a nuclear localized protein that is proposed to regulate proteolysis of cell-cycle transcription factors as a downstream component of another possible auxin receptor, Auxin Binding Protein 1 (ABP1) (Grones and Friml, 2015). Until now, ABP1 is the only identified PM localized candidate auxin receptor, and emerging evidence suggest a pivotal role of ABP1 both in rapid cellular and in transcriptional responses (Bargmann and Estelle, 2014; Grones and Friml, 2015). Recent data, however, question the central role of ABP1 as membrane-localized receptor in auxin signalling (Gao et al., 2015; Habets and Offringa, 2015). Even

though the exact role of ABP1 in auxin signalling still needs to be clarified, it is likely that there are auxin receptors close to or at the plasma membrane that mediate rapid transcription-independent responses to auxin.

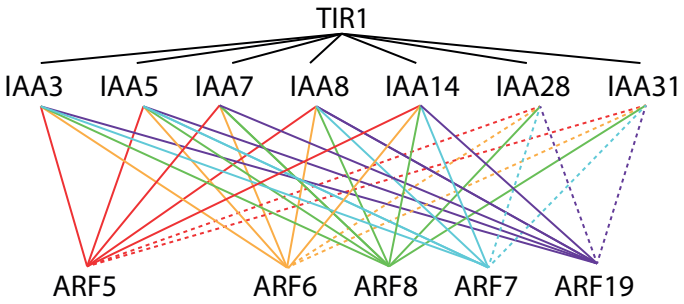

FIGURE 5 - An example of the complexity of auxin signalling.
Protein-protein interactions between TIR1 and Aux/IAAs, and between Aux/IAAs and the activating ARFs. For simplicity, interactions are only displayed for activating ARFs. The interactions are based on Y2H assays of Calderon-Villalobos et al. (2012) and Piya et al. (2014). Solid and broken lines indicate respectively strong and weak interactions.

The role of auxin during somatic embryogenesis

Synthetic auxins are notorious for their use as herbicides, killing dicot weeds, but are also famous for their use in tissue culture to induce the regeneration of shoots, adventitious roots, or somatic embryos. The success of 2,4-D application in somatic embryo culture is thought to lie in its ability to accumulate in plant cells to high levels, caused by its stability in tissue culture, and by the fact that it is not an efficient substrate for the PIN auxin efflux carriers (Delbarre et al., 1996; Petrasek, 2006). Moreover, based on its higher binding affinity to specific TIR1/AFB-Aux/IAA co-receptor pairs, 2,4-D is likely to induce a specific auxin response. There are several factors that should however be taken into account when investigating the auxin response induced by 2,4-D. Firstly, the molecular and genetic response to 2,4-D in cotyledons of the IZE that induce the transition to cells with embryo identity. Secondly, the response is likely to be cell- and tissue-type-dependent, as SE can be induced directly (in the epidermis, adaxial) or indirectly (in the mesophyll, abaxial). Thirdly, once cells with embryo identity have been established, the auxin mechanism will evolve to different responses that further coordinate the development of the somatic embryo.
Below I will discuss the effects of 2,4-D with respect to auxin transport, homeostasis and response during SE.

Auxin transport and homeostasis during SE

SE is induced by the synthetic auxin analogue 2,4-D. For the process to be induced at the molecular genetic level, 2,4-D uptake in cells is required followed by changes in auxin transport and homeostasis. Similar to the natural IAA, 2,4-D is thought to be imported into the cell by AUX1 (Marchant et al., 1999; Yang et al., 2006). However, while IAA is efficiently transported out of the cell by PIN and ABCB proteins (Friml, 2003; Tanaka et al., 2006), 2,4-D is a poor substrate for these auxin efflux carriers (Delbarre et al., 1996; Petrasek, 2006). However, experiments in *Schizosaccharomyces pombe* suggest that 2,4-D can be exported by specific PIN proteins, for example PIN2 and PIN7, but not by PIN1 (Yang and Murphy, 2009). Alternatively, evidence came forward that a member of the ABCG family of transporters ABCG37/PDR9 can function as a 2,4-D efflux transporter (Ito and Gray, 2006). Together, these characteristics allow 2,4-D to accumulate to higher amounts within plant cells compared to IAA, which may explain its toxic effects as herbicide and its efficient SE-inducing effects (Song, 2014). A few studies have shown that genotypes impaired in SE have a lower uptake of 2,4-D and show modified endogenous auxin levels (Ceccarelli et al., 2002; Su et al., 2009; Bai et al., 2013). From this I can conclude that SE induction depends on a strong auxin response. This is similar to the general assumption that an auxin maximum establishes directional growth and polarity of an organ (Bohn-Courseau, 2010), and previous reports have indicated that an auxin maximum is the main driver of SE (Michalczuk et al., 1992; Pasternak, 2002; Gaj et al., 2005; Su et al., 2015). However, somatic embryos have been shown to arise from regions of lower auxin response that co-express PIN1 and PIN4 (Bassuner et al., 2006; Su and Zhang, 2009), suggesting that a change from auxin maximum to minimum is required. Auxin response minima have been shown to contribute to organ formation in combination with oscillating mechanisms and that these are dependent on auxin transport and homeostasis (Wang et al., 2014; Xuan et al., 2015). As PINs do not transport 2,4-D efficiently, it is unlikely that they generate these auxin response minima during SE initiation. More likely, PINs are involved in the further development of the newly initiated somatic embryos, similar to zygotic embryogenesis (Friml et al., 2003; Weijers et al., 2005; Furutani, 2004). However, biosynthesis of auxin via YUCCAs has been shown to be required, as *yucs loss-of-function* mutants, *yuc1 yuc2 yuc4 yuc6* were deficient in SE and their expression was induced in SE cultures (Bai et al., 2013). Based on this observation, YUC-mediated auxin biosynthesis might be required for the dynamic auxin maxima and minima. Alternatively, it may merely provide the auxin for the PAT driven somatic embryo development (Robert et al., 2013). It will be interesting

to see what exactly generates the auxin response minima during SE initiation, and when endogenous auxin biosynthesis becomes required for SE.

The auxin response during SE

As previously described, the auxin response is elevated during SE (Pasternak, 2002; Michalczuk et al., 1992; Simon et al., 2013; Song, 2014). The dichlorophenyl ring and the two chlorines of 2,4-D structurally mimic the two rings of IAA and also fit into the TIR1/AFB-Aux/IAA auxin binding pocket, but the interaction is weaker than with IAA (Tan et al., 2007; Calderón Villalobos et al., 2012). Genetic evidence for 2,4-D mediated TIR1/AFB responses comes from the finding that the *tir1-1* mutant is resistant to 2,4-D-induced auxin responses in the root (Ruegger et al., 1998; Parry et al., 2009). In addition, mutations in *afb1*, *afb2* or *afb3* are sensitive to 2,4-D, suggesting that TIR1 is the main co-receptor for 2,4-D in the root (Parry et al., 2009). The single *tir1-1* mutant does not have a discernible zygotic embryo phenotype (Dharmasiri et al., 2005a), but in 2,4-D induced somatic embryo cultures it was described that mutations in *TIR1* are partially impaired in SE (Gliwicka et al., 2013; Su et al., 2009). Furthermore, recent transcriptome datasets reveal that multiple ARFs (*ARF5/MP, ARF6, ARF10, ARF16, ARF17*) and Aux/IAAs (*IAA16, IAA30, IAA29, IAA31*) are differentially expressed during SE. Phenotypic analysis of the *arf* loss-of-function mutants was not described, but all four corresponding *aux/iaas* loss-of-function mutants showed a reduced SE efficiency (50-70% versus 90% in wild-type), and *iaa30* even showed a reduced SE productivity (3 somatic embryos per IZE versus 4 in wild-type) (Gliwicka et al., 2013; Wickramasuriya and Dunwell, 2015).

The extensive transcriptome analysis by Gliwicka et al. (2013) shows that most of the transcription factor genes that show enhanced expression during SE are auxin-related. The *PUCHI* gene came forward as one of the highest expressed transcription factors in the early stages of SE. *PUCHI* acts together with *LATERAL ORGAN BOUNDARIES DOMAIN 16* and *18* (*LBD16*, *LBD18*), which are downstream targets of *ARF7* and *ARF19* (Kang et al., 2013). *ARF7* and *ARF19* are part of the well-established *SOLITARY ROOT(SLR)/IAA14 – ARF7 – ARF19* module involved in lateral root initiation (Fukaki et al., 2002; De Smet, 2012; Guseman et al., 2015). As mentioned earlier, callus and lateral root development are under the same genetic control and because the exact identity of embryogenic callus is still unknown, it will be interesting to learn from other callus regeneration systems (Sugimoto et al., 2010).

Taken together, multiple independent researchers have shown that 2,4-D induces a TIR1/AFB mediated auxin response and several auxin signalling components have been identified in SE. The question remains, however, which TIR1/

AFB – Aux/IAA – ARF combination is responsible for 2,4-D-induced SE initiation, including the switch from somatic to embryo cell fate identity.

Transcription factor-induced SE and intersection with auxin

In *Arabidopsis*, SE can be induced in the absence of 2,4-D by ectopic expression of specific transcription factors. *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *BABY BOOM, RKD1/GRD,* and *WUSCHEL* (*WUS*) induce somatic embryo formation on seedlings in the absence of exogenous hormones (Lotan et al., 1998; Stone et al., 2001; Boutilier et al., 2002; Zuo et al., 2002; Köszegi et al., 2011). In this section we will highlight research on these genes in relation to SE and their link with auxin homeostasis and signaling.

LEC1 encodes a protein with sequence similarity to the B-domain of the HEME-ACTIVATED PROTEIN3 (HAP3) subunit of the CCAAT-box binding factor (CBF) (Lotan et al., 1998). The *HAP3* gene family in *Arabidopsis* comprises 36 members with the most closely related *LEC1-LIKE (L1L)* factor also being involved in seed development (Junker et al., 2012). *LEC2* and *FUS3* encode proteins with a so-called basic region 3 (B3 domain), a DNA binding motif unique to plant transcription factors with a role in developing seeds and is conserved among (*VIVIPAROUS1*) *VP1/ABI3* proteins (Luerssen et al., 1998; Stone et al., 2001). *LEC1*, *LEC2*, *ABI3*, *FUS3* and *L1L* constitute the *LAFL* gene network, key regulators of embryo morphogenesis, seed maturation and are down-regulated during seed germination (West et al., 1994; Stone et al., 2001; Braybrook et al., 2006; Braybrook and Harada, 2008; Jia et al., 2014). Defects associated with disruptive mutations in *LAFL* genes are aberrant cotyledon development, defects in storage macromolecule accumulation, altered desiccation tolerance and premature germination (Keith et al., 1994; Meinke et al., 1994; West et al., 1994; Parcy et al., 1997; Lotan et al., 1998; Stone et al., 2001). Moreover, *loss-of-function* mutants *lec1*, *lec2* and *fus3* have lost the capacity to perform SE. Fewer explants respond as well as less somatic embryos are formed per explant. Instead the explant produces watery callus and root hairs (Gaj et al., 2005). This is in line with the phenotypes of constitutively overexpressed *LEC1* and *LEC2* that leads to spontaneous SE on the shoot apical meristem and cotyledons of germinating seedlings (Lotan et al., 1998; Stone et al., 2001). However, ectopically expressed *FUS3* results in cotyledon-like leaves (Gazzarrini et al., 2004), presumably by holding the post embryonic tissues in a specific phase of the maturation process which benefits the embryogenic capacity. It was described that *LEC1* induced embryonic differentiation is dependent on ABA levels, and subsequent activation of both *LEC1* and *LEC2* leads to en-

dogenous auxin production which results in reprogramming of epidermal cells generating callus and SE (Stone et al., 2008; Junker et al., 2012). Moreover, Su et al. (2012) showed that disruption of ABA levels can alter auxin biosynthesis and transport subsequently affecting SE negatively.

Besides regulating seed maturation genes, the *LEC1* and *LEC2* transcription factors also regulate auxin biosynthesis and –signalling genes (reviewed by Jia et al., 2014) suggesting an additional mechanism for somatic embryo induction by these LAFL proteins. Both DNA binding and transcriptional regulation has only been demonstrated for a number of these target genes. LEC1 binds to the promoter of *YUC10* and activates its expression in seedlings, but only in combination with ABA (Junker et al., 2012). Similarly, LEC2 binds and activates the expression of *YUC4* in seedlings (Stone et al., 2008). LEC2 also rapidly upregulates *YUC2* and *IAA30*, but it is not clear whether these genes are direct or indirect LEC2 targets (Braybrook et al., 2006; Junker et al., 2012). Notably, indirect measurements of auxin levels through colorimetric analysis showed that auxin levels were increased upon *LEC1* and *LEC2* overexpression in seedlings, supporting their role in SE induction through auxin (Wójcikowska et al., 2013; Junker et al., 2012).

Ectopically expressed *LEC2* can outweigh for lower dosages of 2,4-D (0.1-1.0μM) or for auxins that are less efficient in somatic embryo induction, such as IAA or NAA, indicating that cells become more sensitive to auxin or that *LEC2* induces increased production of endogenous IAA (Wójcikowska et al., 2013). Conversely, overexpression of *LEC2* in combination with a 2,4-D concentration normally used to induce SE (5μM) is detrimental for somatic embryo production, with callus being produced instead of somatic embryos (Ledwoń and Gaj, 2009; Wójcikowska et al., 2013). In two different SE induction systems it was independently shown that *YUC1*, *YUC4* and *YUC10*, are differentially expressed during SE (Wójcikowska et al., 2013; Bai et al., 2013). Additionally, the 1-step protocol SE induction system showed that *TAA1* was expressed as well and the 2-step protocol showed *YUC2* and *YUC6* are also upregulated during SE (Wójcikowska et al., 2013; Bai et al., 2013). Both systems indicate that it results in enhanced endogenous auxin production. In the primary system, it was shown that *LEC2* expression clearly enhances auxin sensitivity in IZE cultures, possibly through IAA30, but the direct regulation of auxin biosynthesis or -signaling by *LEC2* remains to be solved (Braybrook et al., 2006; Wójcikowska et al., 2013).

As earlier mentioned, ectopically expressed *BBM* can also induce spontaneous SE formation. *BBM*, also known as *PLETHORA4 (PLT4)*, is one of the *PLT* family members and is expressed in developing embryos and seeds. Other *PLT* family members such as *PLT3*, *5* and *7* have been shown to coordinate shoot regenera-

tion, rhizotaxis and regulate auxin biosynthesis through *YUC1* and *YUC4* in phyllotaxis (Kareem et al., 2015; Hofhuis et al., 2013; Pinon et al., 2013). Moreover, *PLT1* and *PLT2* have not only been shown to be involved in the root development but also in callus regeneration (Galinha et al., 2007; Feng et al., 2012). The role of *PLTs* in embryogenesis was clarified through the aberrant embryonic phenotype of combined mutations in *plt1*, *plt2*, *plt3* and *bbm* (Galinha et al., 2007; Aida et al., 2004) and through dose dependent ectopically expressed PLT2 and BBM proteins that showed increase in SE capacity upon increased dosages (Horstman, 2015). Both PLT2 and BBM activate regulators of LAFL proteins (Horstman, 2015) and it was found that BBM can bind to auxin biosynthesis genes *YUC3* and *YUC8* and indirectly activate*YUC4* by binding to *STYLISH1* (*STY1*). Also auxin transport gene targets were found, i.e. efflux carriers *PIN1*, *PIN4* and *ABCB19* (Heidmann, 2015), but the exact role of BBM in regulating auxin biosynthesis and -transport during SE remains to be solved.

In conclusion, the experimental data discussed in this Chapter shows that in both 2,4-D- and TF-induced SE, auxin transport, -biosynthesis and -signaling play pivotal roles in establishing the dynamic auxin responses that are required for the initiation and development of somatic embryos.

Outline of the thesis

SE is a highly complex regeneration process that consists of multiple steps i.e. the reversion of cell fates from 'late' to adapting the new cell fate of 'early embryonic', followed by establishing the embryonic body of somatic embryos and development thereof. Auxin is involved in many plant development and regeneration processes and multiple lines of evidence, as described in this Chapter, point to an instructive role for auxin during SE.

However, despite the multiple lines of evidence, the exact genetic and molecular mechanism by which 2,4-D induces SE initiation has not been elucidated yet and this was the key objective of the research described in this PhD thesis. A better understanding of the process will enable us to induce SE more efficiently for high-throughput clonal propagation of crops.

To study cell redifferentiation, robust protocols and cellular markers are required. In Chapter 2 we first established a robust protocol for the efficient and reproducible induction of SE from *Arabidopsis* IZEs. The *pWOX2::NLS-YFP* reporter was used to mark cells that have adapted an early embryo cell fate and the *DR5::RFP* auxin response reporter was used to simultaneously monitor auxin responses. With these tools we demonstrated that for IZEs harvested 12 days after pollination

(12 dap) the SE process was most efficient and productive, and that SE initiation occurred after 5 to 7 days during SEIM incubation. As previously described, we confirmed that an auxin maximum is created but that this is followed by a local auxin minimum, in regions where somatic embryos arise. Through combined studies of mutants and inhibitors of polar auxin transport we showed that this is not caused by PIN auxin efflux carriers, but rather by auxin influx carriers AUXIN1/LIKE-AUXIN1-3 (AUX1/LAX1-3). Analysis of AUX1/LAX1-3 showed a high level of redundancy and that they create an auxin minimum possibly by retaining endogenous auxin in cells surrounding SE initiation regions.

In Chapter 3 we further investigated at what time point auxin biosynthesis is required during SE. We demonstrated that the tryptophan (Trp) to indole-3-pyruvic acid (IPyA) conversion mediated by the TAA1/TAR1-4 enzymes is contributing to the dynamic auxin response by increasing auxin levels in the entire IZE and that this is also crucial for cell development during SE initiation. By using the embryo cell fate marker *WOX2* and auxin response marker *DR5* we could show that the local auxin minimum is absent when the TAA1/TAR1-4 activity is inhibited by L-kynurenine, indicating that this enzymatic step is crucial to cell- development and redifferentation to an early embryo cell fate. Furthermore, we showed that the YUCCA family proteins, responsible for the IPyA to indole-acetic acid (IAA) conversion, function redundantly during SE and that they are crucial for survival of the IZE explants in tissue culture, but not for SE initiation.

In Chapter 4, we used the tools presented in Chapter 2 and the auxin antagonist auxinole, that blocks the binding of auxin to the TIR1/AFB-Aux/IAA co-receptors, to show that SE initiation is extremely dependent on the dynamic auxin responses established through TIR1/AFB co-receptors.. By screening reporters for the 23 *ARFs* we found that all activating *ARF* genes (*ARF5, 6, 7, 8* and *19*), are involved in SE initiation. A significant reduction in SE efficiency was observed for the *arf7 arf19* loss-of-function mutant, the *RPS5a>>miRNA167a* line in which *ARF6* and *ARF8* are knocked down, and interestingly also in the dominant negative solitary root mutant *slr-1*. These findings strongly suggest for an overlap between lateral root founder cell specification and embryonic founder cell specification. As lateral root and callus development also share similar molecular pathways, we used this fact to demonstrate that the auxin response mutants that were blocked in SE also showed a reduction in 2,4-D-induced root callus development. This phenotype seems to provide a novel and simple screen to quickly identify new candidate genes involved in SE initiation.

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